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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

INVENTOR(S)

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☒ *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

MUSCLEBLIND GENE THERAPY FOR RNA-MEDIATED DISEASE

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☒ Drawing(s) *Number of Sheets* 4

☒ Other (specify) 1 pg. of claims

☐ Application Data Sheet. See 37 CFR 1.76

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☐ No.

☒ Yes, the name of the U.S. Government agency and the Government contract number are: National Institutes of Health, Grant No. U54-NS48843

Respectfully submitted,

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REGISTRATION NO. *(if appropriate)* 33,928

Docket Number: 49163 (60677)

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Additional Page

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Docket Number	49163 (60677)	Type a plus sign (+) inside this box →	+

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Number 2 of 2

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Serial No.	Filing Date	Examiner	Group Art Unit

Invention: **MUSCLEBLIND GENE THERAPY FOR RNA-MEDIATED DISEASE**

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49163-60677

APPLICATION FOR UNITED STATES PROVISIONAL APPLICATION

MUSCLEBLIND GENE THERAPY FOR RNA-MEDIATED DISEASE

BY

Maurice S. Swanson

Rahul N. Kanadia

And

Charles A. Thornton

This invention was supported in part by a grant from the National Institutes of Health (U54-NS48843). The United States Government has rights in the invention.

TECHNICAL FIELD

[0001] The field of the invention relates to the use of recombinant adeno-associated virus (rAAV) in treating neurological and neuromuscular diseases. In particular, the methods and compositions are directed to ameliorating the symptoms of diseases caused by microsatellite repeat expansion mutations.

BACKGROUND OF THE INVENTION

Microsatellite Expansion Diseases

[0002] Aberrant expansion of microsatellites in DNA is associated with a number of neurological and neuromuscular diseases (see O'Donnell, 2002). These diseases are caused by microsatellite repeat expansions in coding and non-coding regions. The characterized coding region expansion diseases include Dentatorubral pallidoluysian atrophy (DRPLA), Huntington chorea (HD), Oculopharyngeal muscular dystrophy (OPMD), Spinobulbar muscular atrophy (SBMA), and Spinocerebellar ataxia types 1, 2, 3, 6, 7, and 17 (SCA1, SCA2, SCA3, SCA6, SCA7, SCA17). The characterized non-coding region expansion diseases include Fragile XA, Fragile XE, Friedrich's ataxia, Myotonic Dystrophy type 1 (DM1), Myotonic Dystrophy type 2 (DM2), and Spinocerebellar ataxia types 8, 10, and 12 (SCA8, SCA10, SCA12). Huntington's disease-like type 2 (HDL2) is likewise caused by a microsatellite expansion.

[0003] Microsatellite expansion diseases have been most commonly associated with trinucleotide expansion mutations. In fact, at least 16 of the microsatellite expansion diseases reported to date have been characterized as trinucleotide expansion diseases. More recently, however, microsatellite expansion diseases have also been associated with tetranucleotide and even pentanucleotide expansion mutations. Disease severity and age of onset have both been related to the size of the expansion mutation, eventually leading to muscle weakness and premature cataract formation, and, in severe cases, to hypotonia, muscle heart block, and nervous system dysfunction (for a review, see Korade-Mirnics, *et al.*, 1998).

[0004] Myotonic dystrophy (dystrophia myotonica, DM) is a multisystemic, dominantly inherited disorder often characterized by myotonia, or, delayed muscle relaxation due to repetitive action potentials in myofibers, and muscle degeneration. Manifestations of DM may also include heart block, ocular cataracts, hypogonadism, and nervous system dysfunction.

[0005] Myotonic dystrophy type 1 (DM1) is caused by a trinucleotide $(CTG)_n$ expansion (n=50 to >3000) in the 3'-untranslated region (3'UTR) of the *dystrophia myotonica protein kinase (DMPK)* gene. Myotonic dystrophy type 2 (DM2) is caused by a tetranucleotide $(CCTG)_n$ expansion (n=75 to ~11,000) in the first intron of *zinc finger protein 9 (ZNF9)* gene (Ranum and Day, 2002).

[0006] Both DM1 and DM2 mutant transcripts accumulate as foci within muscle nuclei (Liquori, *et al.*, 2001). An indication that these transcripts are pathogenic comes from studies on HSA^{LR} mice, which express a large CTG repeat in the 3'-UTR of a human skeletal actin transgene (Mankodi, *et al.*, 2000). These transgenic mice develop myonuclear RNA foci, myotonia, and degenerative muscle changes similar to those seen in human DM. The myotonia in HSA^{LR} mice is caused by loss of skeletal muscle chloride (ClC-1) channels due to aberrant pre-mRNA splicing (Mankodi, *et al.*, 2002). Similar ClC-1 splicing defects exist in DM1 and DM2. However, the connection between accumulation of mutant DM transcripts in the nucleus and altered splice site selection has not been established (Faustino and Cooper, 2003).

Muscleblind Proteins

[0007] Proteins in the muscleblind-like (MBNL) family bind to expanded CUG repeats *in vitro* and colocalize with mutant DM and HSA^{LR} transcripts *in vivo*. Human muscleblind genes *MBNL1*, *MBNL2*, and *MBNL3* are homologous to the *Drosophila* gene muscleblind, which is essential for muscle and eye differentiation. *MBNL1*, the major *MBNL* gene expressed in human skeletal muscle, encodes multiple protein isoforms, including some that bind to expanded CUG repeats (41 to 42 kD) and others that fail to bind (31 kD isoform), generated by exon 3 skipping. Expression of CUG and CCUG expansion RNAs induces MBNL recruitment into nuclear RNA foci, but there is no evidence that this relocalization results in muscleblind depletion and functional impairment.

BRIEF SUMMARY OF THE INVENTION

[0008] The invention includes a mouse model for myotonic dystrophy, which has been shown to respond to a rAAV-based gene therapy treatment. Using the HSA^{LR} DM mouse model, administration of a rAAV-MBNL1/41 composition showed functional reversal of the DM phenotype.

[0009] The invention is additionally directed to the use of recombinant adeno-associated viruses (rAAV) that express muscleblind proteins (MBNL) to treat neurological and neuromuscular diseases caused by microsatellite repeat expansion mutations.

[00010] A mouse *Mbnl1* gene knockout model, which replicates many of the clinical features of DM, including myotonia and ocular cataracts, has been made (Kanadia, *et al.*, 2003). This provides the basis for considering the possibility that Mbnl overexpression in muscle and other disease-affected tissues will reverse the deleterious effects of mutant DM1 and DM2 gene expression. To test this possibility, recombinant adeno-associated viruses were constructed to express human MBNL1 (rAAV-MBNL1(rAAV-MBNL1/41)). The rAAV-MBNL1/41 viruses were injected into the tibialis anterior (TA) muscles of a transgenic model for DM that expresses a human skeletal α-actin transgene carrying 250 CTG repeats (HSA^{LR}). The HSA^{LR} mouse model develops myotonia and muscle degeneration similar to muscle abnormalities seen in DM patients. Using this model, a functional reversal of a DM-related phenotype has been demonstrated, namely, reversal of mis-splicing of the Clcn1 skeletal muscle chloride channel which results in myotonia.

[00011] The results in the disclosed mouse model indicate that rAAV-MBNL gene therapy will be useful in treating myotonic dystrophy in humans, as well as other neurological (*e.g.*, SCA8) and neuromuscular diseases caused by microsatellite repeat expansion mutations.

BRIEF DESCRIPTION OF THE FIGURES

[00012] FIG. 1A shows targeted disruption of *Mbnl1*. The illustration includes C57BL/6J *Mbnl*1 exon organization (open boxes, UTRs black boxes, open reading frame) together with the 129S1/*Svlm]* insert (black rectangle), the 129 genomic region with *Eco*RV (E) (E site in C57BL/6] shown by black box with white E), *Xba*1 (X), and *Bam* HI (B) sites, the targeting

construct with a thymidine kinase marker (TK), floxed (black triangles, *lox*P sites), neomycin cassette (stippled box with white N), the 129 region (thick black line) and locations of hybridization probes I and II.

[00013] FIG. 1B is a genomic analysis of *Mbnl*1 mice with the use of probe 1. The 11-kb *Eco*RV fragment is derived from C57BL/6; the mutant is 6.5 kb.

[00014] FIG. 1C shows loss of *Mbnl1* E3 expression in $Mbnl1^{\Delta E3/\Delta E3}$

[00015] FIG. 1D is an immunoblot analysis (total spleen protein) showing absence of Mbnl1 41-42kD proteins in $Mbnl1^{\Delta E3/\Delta E3}$.

[00016] FIG. 2A shows an electromyograph (EMG) of *Mbnl1* wild-type and mutant knockout vastus muscle. The arrow (top panel) indicates normal EMG electrode insertional activity in wild-type muscle, whereas insertion triggers myotonic discharges in $Mbnl1^{\Delta E3/\Delta E3}$ muscle (bottom panel).

[00017] FIG. 2B shows ClC-1 splicing in DM mouse models. Functional chloride channels are produced when Clcn1 exons 6, 7 and 8 are spliced directly together, whereas isoforms that include cryptic exons 7a or 8a encode truncated non-functional proteins. Clcn1 exons 7 to 8 are illustrated (open boxes) with the primer positions indicated via horizontal arrows. Inclusion of exons 7a and 8a occurs at low levels in wild-type (FVB wt, $Mbnl1^{+/+}$) and $Mbnl1^{+/\Delta E3}$ muscle but at increased levels in $Mbnl1^{\Delta E3/\Delta E3}$ and HSA^{LR} muscle.

[00018] FIG. 2C and FIG. 2D depict the loss of ClC-1 protein observed in $Mbnl1^{\Delta E3/\Delta E3}$ vastus muscle. Representative images of sections from 11-week-old mice show reduced ClC-1 immunostaining in $Mbnl1^{\Delta E3/\Delta E3}$ mice (D) relative to wild-type mice (C). Scale bar, 20 μm.

[00019] FIG. 2E and FIG. 2F constitute representative images of sections from 11-week-old mice show equivalent dystrophin (Dys) levels in $Mbnl1^{+/+}$ (E) and $Mbnl1^{\Delta E3/\Delta E3}$ (F) muscle.

[00020] FIG. 2G and FIG. 2H depict abnormal muscle histology. Hematoxylin and eosin (H&E)-stained vastus from wild-type (G) and $Mbnl1^{\Delta E3/\Delta E3}$ (H) mice, showing split myofibers (black arrowhead) and centralized myonuclei (white arrowhead). Scale bar, 30 μm.

[00021] FIG. 2I to FIG. 2L show cataract development. Dilated eyes of 18-week old mice showing a clear wild-type lens (I) but dust-like opacities (white arrowhead) in $Mbnl1^{\Delta E3/\Delta E3}$ mice (K). Center bright spot is the lamp reflection. H&E-stained anterior section (J, L)

highlight increased fragmentation (black arrowhead) and opacities (white arrowhead) in *Mbnl1* $^{\Delta E3/\Delta E3}$ lens (L) compared to wild-type lens (J).

[00022] FIG. 3A shows adult retention of *Tnnt2* exon 5 *Mbnl1* $^{\Delta E3/\Delta E3}$ heart. RT-PCR products with (+) and without (-) exon 5 (black box) are indicated (brackets). Size markers are pBR322 Msp I fragments.

[00023] FIG. 3B shows *Tnnt3* fetal (F) exon inclusion in adult *Mbnl1* $^{\Delta E3/\Delta E3}$. The Tnnt3 protein contains variable N-terminal (alternative splicing of exons 4 to 8 and F) and C-terminal regions (exons 16 and 17) (*23*). RT-PCR (11-week-old mice) of *Tnnt3* exons 2 to 11 (left panel) is shown with alternatively spliced exons 4 to 8 and the fetal (F) exon (black boxes). The F exon contains a *Bsr*BI site (arrowhead) resulting in co-migrating smaller fragments in *Mbnl1* $^{\Delta E3/\Delta E3}$ (right panel).

[00024] FIG. 3C depicts RT-PCR of *Tnnt3* exons 15 to 18 after *Msc*I digestion.

[00025] FIG. 3D shows retention of *Tnnt3* fetal (F) exon in adult DM1 skeletal muscle (left panel). The right panel shows cDNAs containing the F exon (bracket) cleaved with *Bbs*I (arrowhead).

[00026] FIG. 4 shows reversal of the skeletal muscle major chloride channel (*Clcn1*) splicing defect following AAV-MBNL1 injection. + lanes represent AAV-mycMBNL1 injection into the Tibialis anterior (TA) muscles of HSA^{LR} mice, while – lanes represent injection of PBS into the other leg. Boxes indicate *Clcn1* exons. Shown are the normal (bottom, exons 6, 7, 8 spliced directly together) and aberrant (7a, 8a and intron 6 inclusion) splicing patterns. Mice 190 and 191 are uninjected controls.

DETAILED DESCRIPTION OF THE INVENTION

[00027] Use of the terms "an", "a" and "the" and similar terms used in claiming or describing the invention are intended to be construed as including both the singular and plural, unless clearly otherwise indicated or contraindicated. The terms "including", "having" and "containing" are to be construed as open-ended in the same manner as the terms "comprising" or "comprises" are commonly accepted as including but not limiting to the explicitly set forth

subject matter. The term "comprising" and the like are construed to encompass the phrases "consisting of" and "consisting essentially of".

[00028] The methods and processes described herein may be performed in any suitable order unless otherwise indicated or clearly rendered inoperable by a modification in order.

[00029] Limited and narrow interpretation of descriptive language intended to better illustrate the invention is not to be construed as limiting in any way nor to limit the scope of the invention contemplated by the inventors.

[00030] The invention comprises the use of rAAV-expressed muscleblind proteins to treat certain classes of diseases caused by microsatellite repeat expansion mutations. These neurological and neuromuscular diseases include myotonic dystrophy, the most common form of adult-onset muscular dystrophy. A mouse muscleblind knockout model that mimics the symptoms of human myotonic dystrophy has been developed. Using this model, gene therapy utilizing rAAV constructs containing MBNL1 41 kDa isoform cDNA has been shown to ameliorate the previously observed neurological and muscle abnormalities.

[00031] To test whether or not sequestration of MBNL proteins contributes to DM pathogenesis, mice with a targeted deletion of *Mbnl1* exon 3 (E3) (Fig. 1A) were generated. This targeting strategy was predicted to approximate the situation in DM by eliminating synthesis of CUG-binding isoforms (Miller, *et al*, 2000). Genomic blot analysis demonstrated successful deletion of $Mbnl1^{\Delta E3/\Delta E3}$ mice (Fig. 1B). Loss of E3 expression was confirmed by reverse transcription polymerase chain reaction (RT-PCR); primers in exons 3 and 6 were used to amplify a cDNA product from either $Mbnl1^{+/+}$ or $Mbnl1^{+/\Delta E3}$ mice that was absent in $Mbnl1^{\Delta E3/\Delta E3}$ mice (Fig. 1C). As expected, *Mbnl1* expression was not fully eliminated in $Mbnl1^{\Delta E3/\Delta E3}$ mice; RT-PCR products were apparent with primers in constitutively spliced exons 10 and 12, or within exon 13. To confirm elimination of the Mbnl1 41- to 42-kD proteins in $Mbnl1^{\Delta E3/\Delta E3}$ mice, monoclonal antibody 3A4 was used, which recognizes Mbnl1 proteins containing exon 5[MS1]. The 41- to 42-kD isoforms in $Mbnl1^{+/+}$ and $Mbnl1^{+/\Delta E3}$ mice were missing in $Mbnl1^{\Delta E3/\Delta E3}$ (Fig. 1D). Previous studies suggested that elevated levels of another RNA-binding protein, CUGBP1, are responsible for DM-associated RNA splicing changes. However, $Mbnl1^{\Delta E3/\Delta E3}$ mice did not show increased CUGBP1 expression (Fig. 1D).

[00032] *Mbnl1*$^{\Delta E3/\Delta E3}$ mice display overt myotonia beginning around 6 weeks of age. Delayed muscle relaxation was most noticeable after a period of rest and showed improvement during activity. A similar "warm up" phenomenon is characteristic of myotonia in human DM. Electromyographic recordings confirmed myotonic discharges in all *Mbnl1*$^{\Delta E3/\Delta E3}$ mice tested (n = 10) (Fig. 2A). Because myotonia in DM1 and DM2 muscle is associated with aberrant ClC-1 splicing, RT-PCR assays were used to investigate the effect of loss of *Mbnl1* E3 on ClC-1 (encoded by *Clcn1*) expression (Fig. 2B). Remarkably, *Mbnl1*$^{\Delta E3/\Delta E3}$ mice showed abnormal inclusion of *Clcn1* cryptic exons 7a and 8a in a pattern similar to that seen in *HSA*^{LR} mice. Also, some full-length ClC-1 cDNA clones from *Mbnl1*$^{\Delta E3/\Delta E3}$ mice showed abnormal inclusion of intron 2, as has been observed in DM and *HSA*^{LR} muscle. Notably, these abnormal splice isoforms have premature termination codons and do not encode functional chloride channels. By contrast, splicing of the Scn4a sodium channel, the only other ion channel previously associated with myotonia was normal in *Mbnl1*$^{\Delta E3/\Delta E3}$ muscle.

[00033] These results suggested that changes in splice site selection result in the loss of functional ClC-1 from myofiber membranes. Immunofluorescence analysis confirmed a major reduction of ClC-1 protein in *Mbnl1*$^{\Delta E3/\Delta E3}$ muscle relative to the muscle of wild-type sibs (Fig. 2, C and D), whereas the membrane-associated proteins dystrophin (Fig. 2, E and F) and α-sarcoglycan were unaffected. Because abnormalities of ClC-1 splicing in *Mbnl1*$^{\Delta E3/\Delta E3}$ muscle are more pronounced than in *HSA*^{LR} muscle, and considering that *HSA*^{LR} mice have a >80% reduction of chloride conductance, it is likely that myotonia in *Mbnl1*$^{\Delta E3/\Delta E3}$ mice is due to improper ClC-1 pre-mRNA splicing.

[00034] Histological analysis of *Mbnl1*$^{\Delta E3/\Delta E3}$ mice up to 11 weeks of age did not show major degeneration of muscle fibers. Pathological features in *Mbnl1*$^{+/\Delta E3/\Delta E3}$ muscle included an increase in nuclei with an abnormal (central) position and splitting of myofibers (Fig. 2, G and H). Histologic abnormalities were not observed in *Mbnl1*$^{+/+}$ or *Mbnl1*$^{+/\Delta E3}$ muscle. Besides muscle abnormalities, distinctive ocular cataracts that progress from subcapsular "dust-like" opacities to mature cataracts are a prominent DM-associated feature. Similar cataracts were observed in all *Mbnl1*$^{\Delta E3/\Delta E3}$ eyes examined (n = 24; 3 to 8 months old) but not in wild-type siblings (Fig. 2, I to L).

[00035] Abnormal regulation of alternative splicing has been observed in DM1 muscle for cardiac troponin T (TNNT2), insulin receptor (INSR), and ClC-1. Of these, analysis of INSR is uninformative because human patterns of INSR alternative splicing are not conserved in mice. However, *Mbnl1*$^{\Delta E3/\Delta E3}$ adult heart shows abnormal retention of the *Tnnt2* "fetal" exon 5 (Fig. 3A), as was observed for DM1. To determine whether alternative splicing of other genes is disrupted in *Mbnl1*$^{\Delta E3/\Delta E3}$, fast skeletal muscle troponin T (Tnnt3) was assessed. Primers in *Tnnt3* exons 2 and 11 produced a single major RT-PCR product in adult *Mbnl1*$^{+/+}$ and *Mbnl1*$^{+/\Delta E3}$ mice that was undetectable in *Mbnl1*$^{\Delta E3/\Delta E3}$ mice (Fig. 3B). Instead, a cluster of larger cDNAs, all containing a "fetal" (F) exon, was prominent. In contrast, mutually exclusive splicing of *Tnnt3* exons 16 and 17 was unaffected in *Mbnl1*$^{\Delta E3/\Delta E3}$ mice; this finding shows that altered *Mbnl1* expression has specific effects on splice site selection even within the same pre-mRNA (Fig. 3C). Similar alterations of TNNT3 splicing in adult DM1 muscle (Fig. 3D) were found.

[00036] Loss of specific Mbnl1 isoforms that associate with expanded $(CUG)_n$ and $(CCUG)_n$ RNAs is sufficient to cause myotonia, cataracts, and RNA splicing defects that are similar to those seen in DM. Although muscleblind-like proteins may influence gene expression at multiple levels, these proteins may play a direct role in splice site selection. Recent co-transfection analysis in HEK293 cells using a *Tnnt3* mini-gene indicated that the Mbnl1 41 kDa protein regulates alternative splice site choice by binding to a discrete RNA element upstream of the fetal)F) exon. Thus, MBNL proteins bind to distinct RNA sequence elements and influence exon use during splicing.

[00037] Young *Mbnl1*$^{\Delta E3/\Delta E3}$ mice do not develop the severe neonatal muscle weakness associated with congenital DM1, and it is not yet known whether cardiac conduction problems develop in this model. Thus, some aspects of the DM phenotype may not result from loss of *MBNL1* function alone. Additional muscleblind proteins (MBNL2 and MBNL3) are also recruited to nuclear RNA foci. It is contemplated that their sequestration may be required to fully replicate the multisystemic DM phenotype.

Administration of Pharmaceutically Formulated Gene Vectors

[00038] Pharmaceutical preparations of the disclosed gene vectors may be administered intravenously, parenterally or intraperitoneally. Solutions of pharmaceutically acceptable salts can be prepared in water suitable mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations will contain a preservative to prevent growth of microorganisms.

[00039] The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (such as, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, such as, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[00040] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active

ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[00041] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically-active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

[00042] The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

[00043] The composition can be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organics acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

[00044] For parenteral administration in an aqueous solution, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the

present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

[00045] The invention, now described generally and in some detail, will be understood more readily by reference to the following examples, which are provided by way of reference and are in no manner intended to be limiting.

EXAMPLES

Example 1

[00046] The pMbnl1$^{\Delta E3neo}$ targeting plasmid was constructed using pTKflNeo (gift of E. Scott, University of Florida), which contains the Herpes simplex virus - thymidine kinase (HSV-TK) negative selection marker and a loxP-flanked phosphoglycerate kinaseneomycin (PGK-Neo) positive selection cassette. A 2.5 kb *Xbal* fragment (5' arm of homology) corresponding to the upstream region bordering *Mhnl1* exon 3, was inserted 5' of PGK-Neo. For the 3' arm of homology, a 6 kb Mbnl1 *BamHI* fragment was subcloned into pBluescript II KS^{+} (Stratagene, La Jolla, CA), excised with *XhoI/NotI,* and cloned into the *XhoI/NotI* sites of pTKflNeo 3' of PGK-Neo.

[00047] The pMbnl1$^{\Delta E3neo}$ plasmid was linearized with *NotI* and electroporated into CJ.7 ES cells (P.J. Swiatek, T. Gridley, *Genes & Dev.* **7**, 2071 (1993)). ES cells were cultured and selected as described in T. Yang *et al.*, *Nat. Genet.* **19**, 25 (1998). Clones resistant to G418 and FlAU were isolated and screened for homologous recombination by utilizing a forward primer (5'-TGGGATGGAATTGTGGTGTGTTGTTGCTCATG-3') outside the 5' homologous region and a reverse primer (5'-TCCATTTGTCACGTCCTGCACCGACGC-3') in PGKNeo. Amplification (25 cycles) consisted of 98°C for 20 s followed by 68°C for 4 min. Targeted ES cell clones yielded a 2.9 kb PCR product.

[00048] Five ES cell clones (35, 56, 92, 111, and 120) that were positive for homologous recombination were confirmed by genomic DNA blot analysis. Based on restriction map analysis of genomic fragments flanking E3, ES genomic DNA digested with *EcoRV* produces a 16 kb band when a 300 NT*Mbnl1 BamHI/EcoRV* fragment outside the 3' arm of homology is used as a hybridization probe (probe II of Fig. IA). In the targeted allele, a new *EcoRV* site (from pBluescript II KS^+) is introduced, generating a novel 6.7 kb *EcoRV* fragment. All five clones that were positive by PCR were also positive by genomic DNA blot analysis. When the 5' arm of homology (2.5 kb *XbaI* fragment) was used as probe, two bands at 16 kb (wild type) and 7.5 kb (mutant) were detected. To check for additional insertion events in these five clones, PGK-Neo fragment was used as probe on genomic DNA digested with *EcoRV.* A single band at 7.5 kb confirmed the absence of any additional insertion events.

[00049] One ES clone (ES.35) was expanded and transiently transfected with Cre-recombinase to excise PGK-Neo. To detect PGK-Neo loss, forward (5'-C'IACGATGGCTGGCTGCAATATGCCTCACTGTAAG-3') and reverse (5'-GGGTTGAATCTCGTTAGGGACACTGGGTGTCTGTAA-3') primers were used for a PCR screen. PCR was performed for 30 cycles, each cycle consisting of 96°C for 30 see, 60°C for 30 sec and 72°C for 2 min. Clones positive for PGK-Neo deletion yielded a I kb band and cassette excision was confirmed by genomic DNA blot analysis. Utilization of a PCR-generated subfragment of the 5' arm of homology as a hybridization probe yielded *EcoRV* bands at 16 kb and 6.5 kb. The loss of PGK-Neo results in decrease in the size of the mutant allele digested with *EcoRV* from 7.5 to 6.5 kb. The Neo excised allele was designated $Mbnl1^{\Delta E3}$

Example 2

[00050] *Two* $Mbnl1^{+/\Delta E3}$ ES clones (1B3, 2C1) were transferred to 3.5 dpc C57BL/6J blastocysts which were then carried to term by B6D2F1/J recipients. One chimeric male was obtained from each clone. Contribution of CJ.7 (129S1/SvlmJ) ES cells to the germline was determined by mating the chimeric males with C57BL/6J females. Agouti pups in litters sired by the 1B3 chimeric male indicated germline transmission.

[00051] To detect heterozygotes in the F1 population derived from 1 B3, a combination of one forward primer (5'-CTACGATGGCTGGCTGCAATATGCCTCACTGTAAG-3') and two reverse primers [for the mutant allele (5'

[00052] GGGTTGAATCTCGTTAGGGACACTGGGTGTCTGTAA-3']; [for the wild-type allele (5'-TGGCAGACCCTTTGACACCG-3')] were used for PCR. Amplification was performed for 30 cycles, each cycle consisting of 96°C for 30 sec, 60°C for 30 sec and 72°C for 2 min. Heterozygotes were then mated to obtain *Mbnl1* $^{\Delta E3/\Delta E3}$ mice. To confirm loss of exon 3, an RT-PCR strategy was used with the forward primer positioned in exon 3 (5'-TAGTGTCACACCAATTCGGGACACAAA-3') and an exon 6 reverse primer (5'-CCCTTGATGTAATCCATGCAGACAGTGA-3'). Continued transcription of *Mbnl1* in *Mbnl1* $^{\Delta E3/\Delta E3}$ lines was examined using exon 10 forward (5'

[00053] TGCACGGTGCTACGCCAGCC-3') and exon 12 reverse (5'-GTGACGACAGCTCTACATCTGGGTAACA-3') primers as well as exon 13 forward (5'-CCTGCTGCACACTGTTGCCTACAC-3') and reverse (5'-TGTCAGTTCCCTCCCTCACCATGT-3') primers. For amplification, 27 cycles were performed each consisting of 45 sec at 95°C, 45 sec at 55°C and 45 sec at 72°C, followed by a final 10 min extension at 72°C.

Example 3

[00054] Electromyography was performed under general anesthesia (intraperitoneal ketamine, 100 mg/kg; xylazine, 10 mg/kg; and acepromazine, 3 mg/kg) using 30 gauge concentric needle electrodes to examine three hindlimb (tibialis anterior, gastrocnemius, vastus), two forelimb (flexor compartment of distal forelimb, triceps), and thoracolumbar paraspinal muscles. At least 10 needle insertions were performed in each muscle and myotonic discharges were graded on a 4 point scale: 0, no myotonia; 1, occasional myotonic discharge in <50% of needle insertions; 2, myotonic discharge with > 50% of insertions; and 3, myotonic discharge with nearly all insertions. The mean score across all *Mbnl1*$^{\Delta E3/\Delta E3}$ limb muscles was 2.9 in mice age 7 to 11 weeks (n=10). Myotonic discharges were not observed in any muscle in heterozygous *Mbnl1*$^{+/\Delta E3}$ mice (n=9) or wild-type littermates (n=9).

Example 4

[00055] Total cellular RNA was extracted from either quadriceps or heart muscle of *Mbnl1*$^{+/+}$, *Mbnl1* $^{+/\Delta E3}$ and *Mbnl1* $^{\Delta E3/\Delta E3}$ mice by homogenizing the tissues in TRI-REAGENT (Sigma, St. Louis, MO.) according to manufacturer's protocol. First strand cDNA was generated by reverse transcription (RT) using 5 μg of total RNA and SuperScript I I RNase H$^-$ RT (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. For subsequent PCR reactions, 20% of the RT reaction was used as template. Each PCR reaction was spiked with 10 μCi of (α^{32}P)-dCTP (PerkinElmer Life Sciences, Boston, MA). PCR products were resolved on 5-8% nondenaturing polyacrylamide gels followed by autoradiography using Biomax MS film (Eastman Kodak, Rochester, NY).

[00056] For ClC-1 mRNA analysis, the forward primer used corresponded to exon 5 (5'-GGAATACCTCACACTCAAGGCC-3') and the reverse primer to exon 8 (5'-CACGGAACACAAAGGCACTGAATGT-3'). PCR was performed for 27 cycles each consisting of 45 sec at 95°C, 45 sec at 55°C and 45 sec at 72°C, followed by a final 10min extension at 72°C. Full-length ClC-1 cDNA clones were generated from muscle RNA by RT-PCR as previously described (A. Mankodi *et al.*, *Mol. Cell* **10**, 35 (2002)). Sequence analysis of 10 clones from *Mbnl1* $^{\Delta E3/\Delta E3}$ mice revealed 6 clones with inclusion of exon 7a and 2 clones with retention of intron 2. All splice junctions were normal in 10 clones derived from wild-type littermates.

[00057] Tnnt2 was analyzed using exon 2 forward (5'-GCCGAGGAGGTGGTGGAGGAGTA-3') exon 6 reverse (5'-GTCTCAGCCTCACCCTCAGGCTCA-3') and 27 PCR cycles (45 sec at 96°C, 45 sec at 58°C and 45 sec at 72°C, followed by a final 10-min extension at 72°C).

[00058] For mouse Tnnt3, the forward primer overlaps exons 2 and 3 (5'-TCTGACGAGGAAACTGAACAAG-3') while the reverse primer (5'-TGTCAATGAGGGCTTGGAG-3') corresponds to exon 11. For human TNNT3, exon 2 forward (5'-TTCACCATGTCTGACGAGGAAG-3') and exon 10 reverse (5'-CTTCTGGGATCTTAGGAGCAGTG-3') primers were used. For mouse Tnnt3 and human TNNT3, 25 PCR cycles were performed each consisting of 45 sec at 95°C, 45 sec at 50°C and 30 sec at 72°C, followed by a final 10-min extension at 72°C. The same amplification protocol

was used to amplify the mouse Tnnt3 carboxyl terminal region using an exon 15 forward primer (5'- CCTTGTACCAACTGGAGACTGAC-3') and an exon 18 reverse primer (5'-TGATGGTCTCTGCTGCAGTG -3').

[00059] Missense mutations in the Scn4a muscle-specific sodium channel are also associated with myotonia. The Scn4a pre-mRNA has two rare AT/AC splice sites but is not known to undergo alternative splicing. To screen for abnormalities of Scn4a splicing that might contribute to myotonia we carried out RT-PCR analysis of muscle RNA. Partial cDNAs covering the entire Scn4a coding region (GenBank accession# AJ278787) were generated by PCR using the following primers: set 1 exon 1 (GACCTGGAAGCTGGCAAGAAC) to exon 6 (TCCCTTCGTCATTGATGTAGGC); set 2 exon 6 (CCATGAATGACACCAACACCAC) to exon 12 (CTGAGGGTGACGATGAAGCTG); set 3 exon 12 (TCTTCACGGGCATCTTCACTG) to exon 17 (CGCCGCTGTTCAATGTAGATG); and set 4 exon 16 (TGCCTCTATGTGGACATCTCCC) to exon 24 (CGACTCTTTCTTGACGTAGGCG). RT-PCR products from primer sets 1, 2, 3, and 4 was analyzed on 1 % agarose gels before and after restriction digest with *ApaI, NcoI, BspE*I and *BsrGI -HindIII,* respectively. Results show no difference in the length of Scn4a cDNA fragments in $Mbnl1^{+/+}$, $Mbnl1^{+/\Delta E3}$, $Mbnl1^{\Delta E3/\Delta E3}$ or HSA^{LR} mice.

Example 5

[00060] Tibialis anterior (TA) muscles of HSA^{LR} mice were injected with either AAV-mycMBNL1 (+ lanes) or in the other leg with PBS (- lanes) and then assayed three weeks later for recovery of the normal Clcn1 pre-mRNA splicing pattern. Mice 190 and 191 are uninjected controls. The levels of the abnormal splicing products is decreased, while the level of the normal splicing product is increased, following AAV-MBNL1 injection.

Example 6

[00061] Frozen sections (10 μm) of vastus and gastrocnemius muscle were prepared for routine histologic (hematoxylin and eosin, modified Gomori trichrome, periodic acid-Schiff) and histochemical (cytochrome oxidase, acid phosphatase, nicotinamide adenine dinucleotide-tetrazolium reductase, myosin ATPase, succinate dehydrogenase) stains (V. Dubowitz, *Muscle*

Biopsy, A Practical Approach (Bailliere Tindall, London, ed. 2, 1996)). Frozen sections of vastus (6 μm) were immunostained using polyclonal antibodies directed against the C-terminus of ClC-1 (Alpha Diagnostic, San Antonio) or monoclonal antibodies to dystrophin (Dys2) or a-sarcoglycan (NovoCastra, Newcastle upon Tyne) as described in A. Mankodi *et al.*, *Mol. Cell* **10**, 35 (2002).

[00062] For immunological detection of Mbnl1, tissues were placed in homogenization buffer (50 mM Tris-Cl [pH=8.0], 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 6 μg/ml aprotinin, 1 μg/ml leupeptin) and disrupted using a Polytron homogenizer and brief sonication (3 X 5 sec using a microtip sonicator). Following addition of IGEPAL CA-630 (Sigma) to 1%, homogenates were incubated on ice for 15 min, centrifuged at 16,000 X *g* for 10 min. Proteins (30 μg per lane) were detected following SDS-PAGE and immunoblotting using anti-Mbnl1 mAb 3A4 (J. W. Miller *et al.*, *EMBO J.* **19**, 4439 (2000), A. Mankodi *et al.*, *Ann. Neurol.*, in press). Total spleen was analyzed (Fig. ID), because this tissue contains relatively high levels of both Mbnl1and Cugbp1.

[00063] For ocular lens evaluation, mice were sedated using intra-peritoneal injection of 100 mg/kg ketamine (Ketaset, Fort Dodge, IA) and 10 mg/kg xylazine (Xylaject, Phoenix, St Joseph, MO) and anterior chambers and lenses were examined using a slit lamp (Haag Streit, Mason, OH). *In vivo* images were obtained using a Nikon 990 digital camera attached to the slit-lamp. Immediately after euthanasia, globes were enucleated, fixed in paraformaldehyde and embedded in paraffin blocks before being processed overnight in a Shandon Excelsior tissue processor (Thermo Electron, Waltham, MA). Sections (4 μm) were cut using an HM-315 microtome (Richard-Allan, Kalamazoo, MI), dried and H&E stained. Sections were photographed using a Canon EOS D60 digital camera attached to an Olympus Vanox microscope.

Example 7

[00064] Mice, injected with AAV-MBNL1 in one TA while the other side was uninjected, were also tested for myotonia by EMG. Five out of six mice showed elimination of the myotonia in the injected TA muscles but in the uninjected TA of the same animal, robust myotonia (grade level=3) was observed.

REFERENCES

[00065] All references, including patents, published patent applications, scientific publications, and publicly available material cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically set forth as being specifically incorporated by reference in its entirety herein.

Kanadia, R. N., et al., Science, 12 December 2003, Vol. 302, pp. 1978-1980.

Korade-Mirnics, Z., Tarleton, J., Servidei, S., Casey, R.R., Gennarelli, M., Pegoraro, E., Angelini, C. and Hoffman, E.P. Myotonic dystrophy: tissue-specific effect of somatic CTG expansions on allele-specific DMAHP/SIX5 expression. Hum. Mol. Genet. 8, 1017-1023 (1999).

Miller, J. W., et al., The Embo Journal, Vol. 19, No. 17, pp 4439-4448, 2000

Ranum, L.P.W. and Day, J.W., Curr. Opin. Genet. Dev. 12, 266, 2002

UF NEWS, Dec. 11, 2003 (Gainesville, Florida).

[00066] The methods, techniques and compositions disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been illustrated with several examples and preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the methods and compositions, in the steps or in the sequence of steps and in modifications of the compositions without departing from the concept, spirit and scope of the invention. Accordingly, the exclusive rights sought to be patented are as described in the claims below.

WHAT IS CLAIMED IS:

1. A mammalian mouse model for microsatellite repeat expansion disease, comprising a mouse having a deletion of *Mbnl*1 exon 3 (E3) in the mouse genome, wherein said mouse exhibits muscle weakness and ocular cataracts typical of a microsatellite expansion disease symptom in humans.

2. The mouse model of claim 1 wherein the microsatellite repeat expansion disease is caused by a microsatellite expansion in a coding region of DNA.

3. The mouse model of claim 1 wherein the microsatellite repeat expansion disease is caused by a microsatellite expansion in a non-coding region of DNA.

4. The mouse model of claim 1 wherein said mouse exhibits abnormal muscleblind proteins.

4. The mouse model of claim 3 wherein the abnormal muscleblind proteins are associated with myotonia dystrophy.

5. The mouse model of claim 1 wherein said mammalian mouse model exhibits overt myotonia at about six weeks of age.

6. The mouse model of claim 1 wherein said mouse has loss of functional ClC-1 protein encoded by *Clcn1*.

7. A method of ameliorating or eliminating the symptoms of a neuromuscular or neurological condition caused by microsatellite repeat expansion mutation, comprising administering to a mammal in need thereof, a therapeutically effective amount of recombinant adeno-associated virus (rAAV) containing a transgene that expresses either an MBNL1, MBNL2 or MBNL3 protein.

8. The method of claim 7 wherein the transgene is human *MBNL1*.

9. The method of claim 7 wherein the mammal is human.

10. A pharmaceutical composition comprising a recombinant AAV containing a transgene operably linked to a promoter that expresses at least one muscleblind protein.

11. The method of claim 7 wherein the transgene is selected from the group of *Mbnl1, Mbnl2, Mbnl3*

12. The method of claim 11 wherein the transgene is human.

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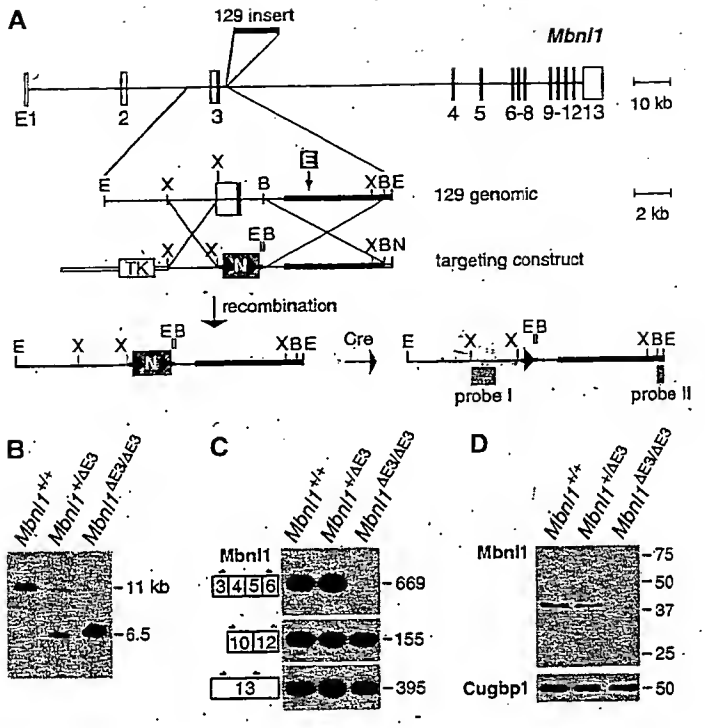
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6-8
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TK
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targeting construct
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probe II
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Mbnl1+/ΔE3
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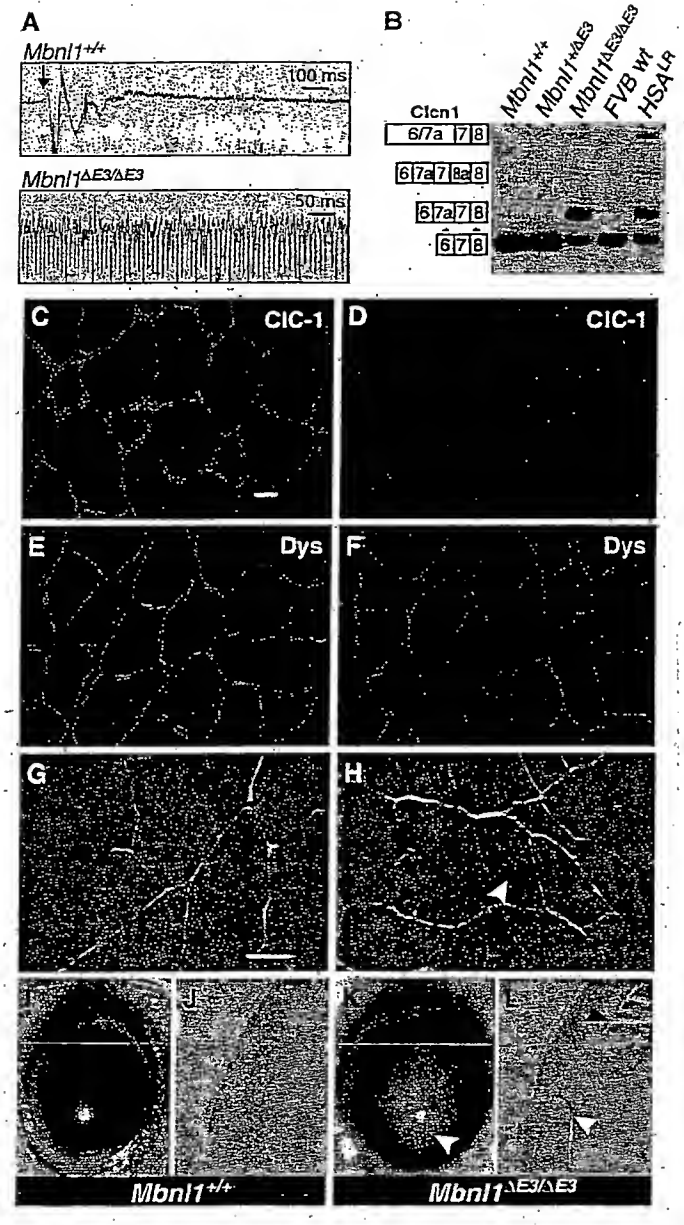
FIGURE 1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FVB wt
HSALR
Clcn1
6/7a 7 8
6 7a 7 8a 8
6 7a 7 8
6 7 8
C
CIC-1
D
CIC-1
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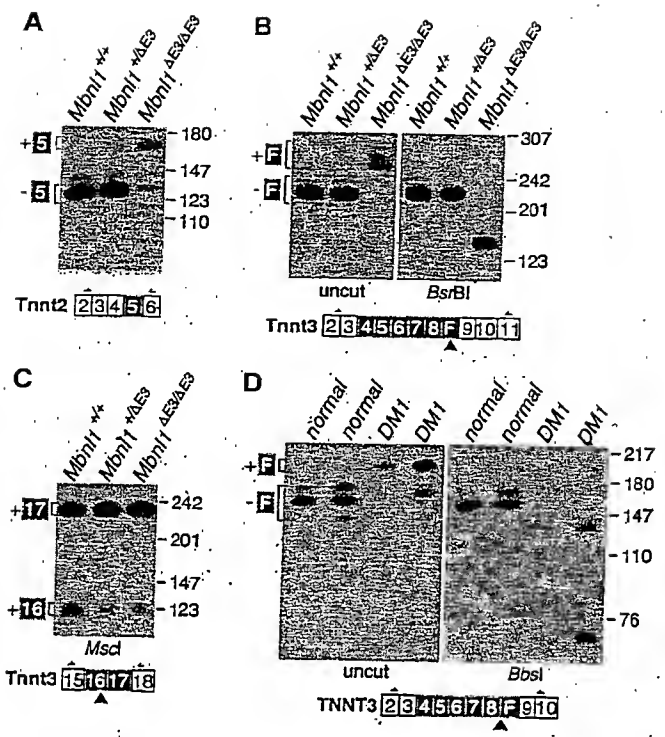
FIGURE 2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-5
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147
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B
Mbnl1 +/+
Mbnl1 +ΔE3
Mbnl1 ΔE3/ΔE3
+F
-F
307
242
201
123
uncut
BsrBI
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Mbnl1 ΔE3/ΔE3
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+16
242
201
147
123
MscI
Tnnt3 15 16 17 18
D
normal
normal
DM1
DM1
normal
normal
DM1
DM1
+F
-F
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uncut
BbsI
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FIGURE 3

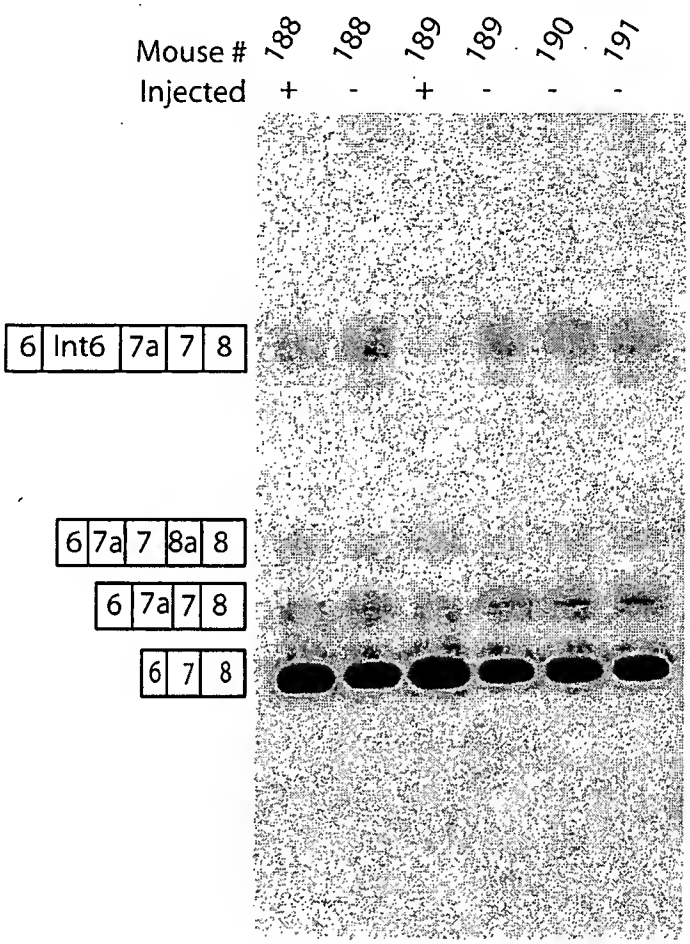

Figure 1. *Reversal of the skeletal muscle major chloride channel (Clcn1) splicing defect following AAV-MBNL1 injection.* Tibialis anterior (TA) muscles of HSA^{LR} mice were injected with either AAV-mycMBNL1 (+ lanes) or in the other leg with PBS (- lanes) and then assayed three weeks later for recovery of the normal Clcn1 pre-mRNA splicing pattern. Boxes indicate *Clcn1* exons. Shown are the normal (bottom, exons 6, 7, 8 spliced directly together) and aberrant (7a, 8a and intron 6 inclusion) splicing patterns. Mice 190 and 191 are uninjected controls. Note that the levels of the abnormal splicing products is decreased, while the level of the normal spliced product is increased, following AAV-MBNL1 injection.

FIGURE 4